

CLAIMS

What is claimed is:

1. A method of generating multiple copies of a nucleic acid sequence of interest, said method comprising the steps of:

5 (a) hybridizing a single stranded target polynucleotide comprising the nucleic acid sequence of interest with a first primer, wherein said first primer is a composite primer comprising an RNA portion and a 3' DNA portion;

(b) optionally hybridizing a polynucleotide comprising a termination polynucleotide sequence to a region of the target polynucleotide 5' with respect to a site for hybridization of the first primer to the target polynucleotide;

10 (c) extending the first primer with a DNA-dependent DNA polymerase to generate a complex comprising a first primer extension product and target polynucleotide;

(d) cleaving the RNA portion from the composite primer in the complex of first primer extension product and target polynucleotide with an enzyme that cleaves RNA from an RNA/DNA hybrid such that another composite primer can hybridize to the target polynucleotide and repeating primer extension by strand displacement to produce a displaced primer extension product;

15 (e) hybridizing a propromoter polynucleotide comprising a propromoter and a region which hybridizes to the displaced primer extension product under conditions which allow transcription to occur by RNA polymerase, such that an RNA transcript comprising sequences complementary to the displaced primer extension products is produced;

(f) hybridizing a second primer to the RNA transcript of step (e);

20 (g) extending the second primer with RNA-dependent DNA polymerase to generate a complex comprising a second primer extension product and the RNA transcript;

(h) cleaving RNA in the complex of step (g) with an enzyme that cleaves RNA in an RNA/DNA hybrid;

(i) hybridizing the single stranded second primer extension product with a propromoter polynucleotide, wherein the propromoter polynucleotide comprises a propromoter and a region which hybridizes to the single stranded second primer extension product under conditions which allow transcription to occur by RNA polymerase, such that an RNA transcript comprising the sequence of interest is produced;

whereby multiple copies of the nucleic acid sequence of interest are produced.

2. A method of generating multiple copies of a nucleic acid sequence of interest, said method comprising the steps of:

(a) combining:

the complex of step (c) of claim 1;

a composite primer that is hybridizable to the target polynucleotide, wherein the composite primer comprises an RNA portion and a 3' DNA portion;

an enzyme that cleaves RNA from an RNA/DNA hybrid;

a propromoter polynucleotide comprising a propromoter and a region which hybridizes to displaced composite primer extension product;

an RNA polymerase;

a second primer that is hybridizable to a sense RNA transcript comprising the sequence of interest;

an RNA-dependent DNA polymerase; and

a propromoter polynucleotide comprising a propromoter and a region which hybridizes to a second primer extension product; and

(b) incubating the mixture of step (a) under conditions that permit primer hybridization and extension, RNA cleavage, displacement of the first primer extension product from the complex of step (c) of claim 1 when its RNA is cleaved and a composite primer binds to the target polynucleotide in the complex, hybridization of a propromoter polynucleotide to a first primer extension product to form a complex comprising a first primer extension product and a propromoter polynucleotide, hybridization of a propromoter polynucleotide to a second primer extension product to form a complex comprising a second primer extension product

and a propromoter polynucleotide, and RNA transcription, whereby multiple copies of the nucleic acid sequence of interest are generated.

3. A method of generating multiple copies of a nucleic acid sequence of interest, said method comprising the steps of:

- 5 (a) combining:
the displaced primer extension product of step (d) of claim 1;
a propromoter polynucleotide comprising a propromoter and a region which hybridizes to displaced first primer extension product;
an RNA polymerase;
10 a second primer that is hybridizable to a sense RNA transcript comprising the sequence of interest;
an RNA-dependent DNA polymerase;
an enzyme that cleaves RNA from an RNA/DNA hybrid; and
a propromoter polynucleotide comprising a propromoter and a region which
15 hybridizes to a second primer extension product; and
(b) incubating the mixture of step (a) under conditions that permit primer hybridization and extension, RNA cleavage, hybridization of a propromoter polynucleotide to a first primer extension product to form a complex comprising a first primer extension product and a propromoter polynucleotide, hybridization of a
20 propromoter polynucleotide to a second primer extension product to form a complex comprising a second primer extension product and a propromoter polynucleotide, and
RNA transcription, whereby multiple copies of the nucleic acid sequence of interest are generated.

4. A method of generating multiple copies of a nucleic acid sequence of interest, said method comprising the steps of:

- 25 (a) combining:
the RNA transcript of step (e) of claim 1;
a second primer that is hybridizable to a sense RNA transcript comprising the sequence of interest;
30 an RNA-dependent DNA polymerase;
an enzyme that cleaves RNA from an RNA/DNA hybrid;

a propromoter polynucleotide comprising a propromoter and a region which hybridizes to a second primer extension product; and

an RNA polymerase; and

(b) incubating the mixture of step (a) under conditions that permit primer hybridization and extension, RNA cleavage, hybridization of a propromoter polynucleotide to a primer extension product to form a complex comprising a primer extension product and a propromoter polynucleotide, and RNA transcription, whereby multiple copies of the nucleic acid sequence of interest are generated.

5. A method of generating multiple copies of a nucleic acid sequence of interest, said method comprising the steps of:

(a) combining:

a first primer, wherein the first primer is a composite primer that is hybridizable to a target polynucleotide, and wherein the composite primer comprises an RNA portion and a 3' DNA portion;

optionally a polynucleotide comprising a termination polynucleotide sequence that is hybridizable to a region of the target polynucleotide which is 5' with respect to hybridization of the composite primer to the target polynucleotide;

a DNA-dependent DNA polymerase;

an enzyme that cleaves RNA from an RNA/DNA hybrid;

a propromoter polynucleotide comprising a propromoter and a region which hybridizes to a first primer extension product;

an RNA polymerase;

a second primer that is hybridizable to a sense RNA transcript comprising the sequence of interest;

an RNA-dependent DNA polymerase; and

a propromoter polynucleotide comprising a propromoter and a region which hybridizes to a second primer extension product; and

(b) incubating the mixture of step (a) under conditions that permit primer hybridization and extension, RNA cleavage, displacement of a first primer extension product from a complex comprising a first primer extension product and target polynucleotide when its RNA is cleaved and a composite primer binds to the target

polynucleotide in the complex, hybridization of a propromoter polynucleotide to a primer extension product to form a complex comprising a primer extension product and a propromoter polynucleotide, and RNA transcription, whereby multiple copies of the nucleic acid sequence of interest are generated.

5 6. The method of claim 1, wherein the 5' RNA portion is adjacent to the 3' DNA portion.

7. The method of claim 1, wherein a plurality of composite primers are used.

8. The method of claim 1, wherein the polynucleotide comprising a termination sequence is a template switch oligonucleotide (TSO).

10 9. The method of claim 8, wherein the TSO comprises a modification in the region which hybridizes to the template, wherein, under a given set of conditions, the TSO binds more tightly to the region as compared to a TSO without the modification.

10. The method of claim 1, wherein the polynucleotide comprising a termination sequence is a blocking sequence.

15 11. The method of claim 10, wherein the blocking sequence comprises a modification in the region which hybridizes to the target polynucleotide, wherein, under a given set of conditions, the blocking sequence binds more tightly to the region as compared to a blocking sequence without the modification.

12. The method of claim 1, wherein the enzyme that cleaves RNA is RNaseH.

20 13. The method of claim 1, wherein the propromoter polynucleotide comprising a propromoter and a region which hybridizes to a displaced first primer extension product is a template switch oligonucleotide (TSO).

25 14. The method of claim 1, wherein the propromoter polynucleotide comprising a propromoter and a region which hybridizes to a single stranded second primer extension product is a TSO.

15. The method of claim 1, wherein the propromoter polynucleotide comprising a propromoter and a region which hybridizes to a displaced first primer extension product is a propromoter template oligonucleotide (PTO).

30 16. The method of claim 1, wherein the propromoter polynucleotide comprising a propromoter and a region which hybridizes to a single stranded second primer extension product is a PTO.

17. A method of generating multiple copies of a nucleic acid sequence of interest, said method comprising the steps of:

(a) hybridizing a single stranded target polynucleotide comprising the sequence of interest with a first primer;

(b) hybridizing a propromoter template switch oligonucleotide (TSO) comprising a propromoter sequence and a region that is hybridizable to a region of the target polynucleotide which is 5' with respect to hybridization of the first primer to the target polynucleotide;

(c) extending the first primer with DNA polymerase such that a first primer extension product comprising a sequence complementary to the propromoter sequence of the propromoter TSO is produced, whereby a complex of first primer extension product, target polynucleotide and propromoter TSO is generated, wherein said complex comprises a double stranded promoter region;

(d) transcribing from the double stranded promoter region with a DNA-dependent RNA polymerase to generate a sense RNA transcript;

(e) hybridizing a second primer to the sense RNA transcript of step (d);

(f) extending the second primer with RNA-dependent DNA polymerase to generate a complex comprising a second primer extension product and an RNA transcript;

(g) cleaving RNA in the complex of step (f) with an enzyme that cleaves RNA in an RNA/DNA hybrid;

(h) hybridizing a single stranded second primer extension product with a propromoter polynucleotide, wherein the propromoter polynucleotide comprises a propromoter and a region which hybridizes to the single stranded second primer extension product under conditions which allow transcription to occur by RNA polymerase, such that sense RNA transcripts comprising the sequence of interest are produced;

whereby multiple copies of the nucleic acid sequence of interest are produced.

18. A method of generating multiple copies of a nucleic acid sequence of interest, said method comprising the steps of:

(a) combining:
the complex of step (c) of claim 17;
an RNA polymerase;
a second primer that is hybridizable to a sense RNA transcript comprising the

5 sequence of interest;

an RNA-dependent DNA polymerase;
an enzyme that cleaves RNA from an RNA/DNA hybrid; and
a propromoter polynucleotide comprising a propromoter and a region which
hybridizes to a second primer extension product; and

10 (b) incubating the mixture of step (a) under conditions that permit primer
hybridization and extension, RNA cleavage, hybridization of a propromoter
polynucleotide to a primer extension product to form a complex comprising a primer
extension product and a propromoter polynucleotide, and RNA transcription,
whereby multiple copies of the nucleic acid sequence of interest are generated.

15 19. A method of generating multiple copies of a nucleic acid sequence of
interest, said method comprising the steps of:

(a) combining:
an RNA transcript of step (d) of claim 17;
a second primer that is hybridizable to the RNA transcript;
20 an RNA-dependent DNA polymerase;
an enzyme that cleaves RNA from an RNA/DNA hybrid;
a propromoter polynucleotide comprising a propromoter and a region which
hybridizes to a second primer extension product; and

an RNA polymerase; and

25 (b) incubating the mixture of step (a) under conditions that permit primer
hybridization and extension, RNA cleavage, hybridization of a propromoter
polynucleotide to a primer extension product to form a complex comprising a primer
extension product and a propromoter polynucleotide, and RNA transcription,
whereby multiple copies of the nucleic acid sequence of interest are generated.

30 20. A method of generating multiple copies of a nucleic acid sequence of
interest, said method comprising the steps of:

(a) combining:
a target polynucleotide;
a first primer which is hybridizable to the target polynucleotide;
a propromoter template switch oligonucleotide comprising a propromoter
5 sequence and a region that is hybridizable to a region of the target polynucleotide
which is 5' with respect to hybridization of the first primer to the target
polynucleotide;
optionally a DNA-dependent DNA polymerase;
an RNA polymerase;
10 a second primer that is hybridizable to a sense RNA transcript comprising the
sequence of interest;
an RNA-dependent DNA polymerase;
an enzyme that cleaves RNA from an RNA/DNA hybrid; and
a propromoter polynucleotide comprising a propromoter and a region which
15 hybridizes to a second primer extension product; and
(b) incubating the mixture of step (a) under conditions that permit primer
hybridization and extension, RNA cleavage, hybridization of a propromoter
polynucleotide to a primer extension product to form a complex comprising a primer
extension product and a propromoter polynucleotide, and RNA transcription,
20 whereby multiple copies of the nucleic acid sequence of interest are generated.
21. The method of claim 17, wherein the target polynucleotide is DNA.
22. The method of claim 17, wherein the target polynucleotide is RNA.
23. The method of claim 17, wherein the first and second primers are the
same.
25 24. The method of claim 17, wherein the first and second primers are
different.
25. The method of claim 17, wherein the first and second primers hybridize to
different complementary sequences.
26. The method of claim 17, wherein the RNA-dependent DNA polymerase is
30 reverse transcriptase.

27. The method of claim 17, wherein the target polynucleotide is RNA, and RNA in the complex of step (c) is cleaved with an enzyme that cleaves RNA in an RNA/DNA hybrid.

5 28. The method of claim 17, wherein the propromoter polynucleotide comprising a propromoter and a region which hybridizes to a second primer extension product is a propromoter TSO.

29. The method of claim 17, wherein the propromoter polynucleotide comprising a propromoter and a region which hybridizes to a second primer extension product is a propromoter template oligonucleotide (PTO).

10 30. The method of claim 1 or 17, wherein at least one type of rNTP used is a labeled rNTP, whereby labeled RNA products are generated.

31. The method of claim 1, wherein the RNA-dependent DNA polymerase and DNA-dependent DNA polymerase are one enzyme.

15 32. The method of claim 17, wherein the DNA polymerase of step (c) is a DNA-dependent DNA polymerase, and wherein the DNA-dependent DNA polymerase and the RNA-dependent DNA polymerase are one enzyme.

33. The method of claim 1 or 17, wherein the RNA-dependent DNA polymerase and enzyme that cleaves RNA from an RNA/DNA hybrid are the same enzyme.

20 34. The method of claim 1, wherein the DNA-dependent DNA polymerase and enzyme that cleaves RNA from an RNA/DNA hybrid are the same enzyme.

25 35. The method of claim 17, wherein the DNA polymerase of step (c) is a DNA-dependent DNA polymerase, and wherein the DNA-dependent DNA polymerase and enzyme that cleaves RNA from an RNA/DNA hybrid are the same enzyme.

36. The method of claim 1, wherein the DNA-dependent DNA polymerase, the RNA-dependent DNA polymerase and the enzyme that cleaves RNA from an RNA/DNA hybrid are the same enzyme.

30 37. The method of claim 17, wherein the DNA polymerase of step (c) is a DNA-dependent DNA polymerase, and wherein the DNA-dependent DNA

polymerase, the RNA-dependent DNA polymerase and enzyme that cleaves RNA from an RNA/DNA hybrid are the same enzyme.

38. A method of sequencing a target polynucleotide, said method comprising (a) amplifying a target polynucleotide by the method of claim 1 or 17 in the presence of a mixture of rNTPs and rNTP analogs such that transcription is terminated upon incorporation of an rNTP analog; and (b) analyzing the amplification products to determine sequence.

39. A method of sequencing a target polynucleotide, said method comprising (a) amplifying a target polynucleotide by the method of claim 1 or 17, wherein RNA transcripts generated from a first primer extension product are amplified in the presence of a mixture of rNTPs and rNTP analogs such that transcription is terminated upon incorporation of an rNTP analog; and (b) analyzing the amplification products to determine sequence.

40. A method of detecting a mutation in a target polynucleotide by single stranded conformation polymorphism, comprising (a) amplifying the target polynucleotide by the method of claim 1 or 17; and (b) analyzing the amplification products for single stranded conformation, wherein a difference in conformation as compared to a reference single stranded polynucleotide indicates a mutation in the target polynucleotide.

41. A method of characterizing a sequence of interest in a target polynucleotide, said method comprising

(i) amplifying a target polynucleotide sequence containing the sequence of interest by the method of claim 1, wherein the sequence of the RNA portion of the composite primer is known, and

(ii) comparing the amplification products if any from step (i) with the amount of amplification products from a reference template

wherein

(1) production of detectably fewer amplification products from the template as compared to the amount of amplification products from the reference template which comprises a region complementary to the RNA portion of the composite primer indicates that the target polynucleotide does not comprise a sequence complementary

to the RNA portion of the composite primer and is a sequence variant with respect to the sequence complementary to the RNA portion of the composite primer; or

(2) production of detectably more amplification products from the template as compared to the amount of amplification products from the reference template which does not comprise a region which is complementary to the RNA portion of the composite primer indicates that the target polynucleotide comprises a sequence complementary to the RNA portion of the composite primer and is not a sequence variant with respect to the sequence complementary to the RNA portion of the composite primer.

42. The method of claim 41, wherein the sequence of the RNA portion of the composite primer comprises a sequence complementary to a wild type sequence, and the sequence of interest is characterized in determining the presence or absence of the wild type sequence.

43. The method of claim 41, wherein the sequence of the RNA portion of the composite primer comprises a sequence complementary to a mutant sequence, and the sequence of interest is characterized in determining the presence or absence of the mutant sequence.

44. The method of claim 41, wherein the sequence of the RNA portion of the composite primer comprises a sequence complementary to an allelic sequence, and the sequence of interest is characterized in determining the presence or absence of the allelic sequence.

45. A method of producing a microarray, comprising (a) amplifying a target polynucleotide by the method of claim 1 or 17; and (b) immobilizing the amplification products on a substrate to fabricate a microarray comprising the amplification products.

46. A method of characterizing a sequence of interest, comprising (a) amplifying a target polynucleotide by the method of claim 30; and (b) analyzing the labeled RNA products.

47. The method of claim 46, wherein step (b) comprises contacting the labeled RNA products with at least one probe.

48. The method of claim 47, wherein the at least one probe is provided as a microarray.

49. The method of claim 48, wherein the microarray comprises at least one probe immobilized on a substrate fabricated from a material selected from the group consisting of paper, glass, plastic, polypropylene, nylon, polyacrylamide, nitrocellulose, silicon, and optical fiber.

50. The method of claim 49, wherein the probe is immobilized on the substrate in a two-dimensional configuration or a three-dimensional configuration comprising pins, rods, fibers, tapes, threads, beads, particles, microtiter wells, capillaries, and cylinders.

51. The method of claim 46, wherein step (b) of analyzing the labeled RNA products comprises determining amount of said products, whereby the amount of the sequence of interest present in a sample is quantified.

52. A method of determining gene expression profile in a sample, said method comprising:

(a) amplifying at least two sequences of interest in the sample using the method of claim 1 or 17; and

(b) determining amount of amplification products of each sequence of interest, wherein each said amount is indicative of amount of each sequence of interest in the sample, whereby the gene expression profile in the sample is determined.

53. The method of claim 52, wherein each target polynucleotide is a cDNA.

54. A system for amplifying a sequence of interest, comprising: (a) a first primer which is a composite primer; (b) a second primer; (c) a DNA-dependent DNA polymerase; (d) an RNA-dependent DNA polymerase; (e) a propromoter polynucleotide; (f) an RNA polymerase; and (g) an enzyme that cleaves RNA from an RNA/DNA hybrid.

55. The system of claim 54, further comprising: (h) a polynucleotide comprising a termination polynucleotide sequence.

56. A system for amplifying a sequence of interest, comprising: (a) a propromoter TSO; (b) a first primer; (c) optionally a DNA-dependent DNA

polymerase; (d) an RNA-dependent DNA polymerase; (e) an enzyme that cleaves RNA from an RNA/DNA hybrid; (f) optionally a second primer; (g) an RNA polymerase.

5 57. The system of claim 54 or 56, wherein the DNA-dependent DNA polymerase and the RNA-dependent DNA polymerase are the same enzyme.

58. The system of claim 54 or 56, wherein the DNA-dependent DNA polymerase and enzyme that cleaves RNA from an RNA/DNA hybrid are the same enzyme.

10 59. The system of claim 54 or 56, wherein the RNA-dependent DNA polymerase and enzyme that cleaves RNA from an RNA/DNA hybrid are the same enzyme.

60. The system of claim 54 or 56, wherein the RNA-dependent DNA polymerase, DNA-dependent DNA polymerase and enzyme that cleaves RNA from an RNA/DNA hybrid are the same.

15 61. A kit comprising a composite primer that is hybridizable to a target polynucleotide, a propromoter polynucleotide, and instructions for amplifying the target polynucleotide according to the method of claim 1.

62. The kit of claim 61, further comprising a polynucleotide comprising a termination polynucleotide sequence.

20 63. The kit of claim 61, further comprising a second primer.

64. A kit comprising a propromoter TSO and a first primer, wherein both are hybridizable to a target polynucleotide, and instructions for amplifying the target polynucleotide according to the method of claim 17.

65. The kit of claim 64 further comprising a second primer.

5 66. A method of determining sequence of a sequence of interest comprising sequencing a nucleic acid amplification product, wherein said nucleic acid amplification product is generated by the method of claim 1 or 17.

10 67. A method of detecting presence of a nucleic acid sequence of interest in a sample, said method comprising detecting presence of the sequence of interest in a nucleic acid amplification product, wherein said nucleic acid amplification product is generated by the method of claim 1 or 17.